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(54) Title: PROCESS FOR THE PRODUCTION OF PROTEIN FIBRES AND PRODUCTS THEREOF

(57) Abstract

The present invention relates to a process for the production of protein fibres comprising fibronectin and/or fibrinogen, improved fibronectin and/or fibrinogen fibres and articles comprising the protein fibres. The present invention also relates to protein fibres comprising fibrinogen and fibronectin for use in wound healing and in the manufacture of medicaments, dressings or devices for use in therapy.

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# PROCESS FOR THE PRODUCTION OF PROTEIN FIBRES AND PRODUCTS THEREOF

The present invention relates to a process for the production of protein fibres comprising fibronectin and/or fibrinogen, improved fibronectin and/or fibrinogen fibres and articles comprising the protein fibres. The present invention also relates to protein fibres comprising fibrinogen and fibronectin for use in wound healing and in the manufacture of medicaments, dressings or devices for use in therapy.

Treatment of severe burns and wounds frequently depends upon use of an autograft technique to replace the damaged skin. This situation is not generally ideal as it increases the trauma to the patient. However, skin replacements have been developed which attempt to mimic the multi-layer structure of the skin, or which form a cellular sheet over a wound. A number of these are collagen based so that a close resemblance is achieved between the replacement tissue and the natural extracellular matrix [1,2,3] Cartilage and vascular grafts are also being developed from a synthetic polymer, poly D lactic acid and polyglycolic acid, [4,5]. These materials have been found to demonstrate good cell adhesion as well as possessing the required mechanical properties for their intended function. Other techniques include extensive cell culture to produce large areas of keratinocytes for transplantation onto the recipient site for skin replacement.

Purified fibronectin has previously been used to form 3-dimensional mats for cell growth. Typically the dimensions of the mats are 2cm x 1cm [6,7]. Such mats are made from an affinity chromotography purified fibronectin solution in a stirred ultrafiltration cell, using a combination of protein concentration and fluid shear. The formed mats consist of a mass of very fine protein strands, which are generally aligned by the shearing effect in the ultrafiltration cell and give the mat an overall orientation. The mats can be used as a template for orientated cell growth and have found functional useage in nerve repair where they have been seen to bridge a 1cm gap in rat sciatic nerve [8]. Fibronectin mats have also been used as depots for growth factors such as nerve growth factor [9,10] as well as a carrier for keratinocytes with potential use in the treatment of epidermolysis bullosa [11].

Furthermore, Wojciak-Stothard, [12,13] report that single fine strands of the purified fibronectin, diameter 0.2-5$\mu$m, were seen to promote the orientation and migration of fibroblasts and macrophage-like cells along their length. Cell alignment and orientation is believed to be a key process in the reduction of wound contracture and the strengthening of wound repair. However, the very small diameter of fibronectin strands described by Wojciak-Stothard make them difficult to handle.

It is therefore among the objects of the present invention to provide an improved process for the production of protein fibres comprising fibronectin and/or fibrinogen and to provide fibronectin and/or fibrinogen fibres with improved properties.

The present invention provides a process for the preparation of a protein fibre comprising fibronectin and/or fibrinogen, comprising the steps of:

- adding fibronectin and/or fibrinogen to a solvent so as to form a fibronectin and/or fibrinogen solution; and
- extruding the solution through an orifice into a coagulation solution so as to form said protein fibre.

Typically, the solution comprising fibronectin and/or fibrinogen is obtained by dissolving protein solids eg a protein precipitate comprising fibronectin and/or fibrinogen in an appropriate solvent.

An impure protein mixture may be used as a starting material since there is no requirement that it need be in a purified form.

It is preferred that the protein solids comprises a mixture of fibronectin and fibrinogen. Generally the ratio of fibronectin to fibrinogen is in the range of 9:1 to 1:9, preferably 7.5:2.5 to 2.5:7.5, more preferably 6.5:3.5 to 3.5 to 6.5. A particularly preferred protein solids comprises fibronectin and fibrinogen in the ratio 6.5:3.5. Other proteins, such as albumin, may be present in the protein solid in smaller proportions.

Generally, the fibronectin and/or fibrinogen may be obtained from any source. Preferably a fibronectin/fibrinogen mixture may be obtained from a cryoprecipitate of blood plasma, particularly human blood plasma, although blood plasma from other mammalian sources, such as bovine, porcine or ovine sources may be used.

A mixture of fibrinogen and fibronectin may be prepared for example from human blood plasma as described in:

Foster, P.R., Dickson, A.J., McQuillan, T.A., Dickson, I.H., Keddie, S. and Watt, J.G. (1982); Control of large-scale plasma thawing for recovery of cryoprecipitate factor VIII. Vox Sanguinis, 42: 180-189.

Alternatively, the isolation of fibronectin and fibrinogen from human plasma may be derived from a so-called "Cohn fraction I" for which the general method for preparation is described in:

Kistler, P. and Friedli, H. (1980) Ethanol precipitation. In "Methods of Plasma Protein Fractionation" (Ed. Curling, J.M.) Pub. Academic Press, London, pp.3-15.

It may be possible to produce and use plasma protein fractions containing additional protein fractions as well as that known as Cohn fraction I. For example, during plasma fractionation a composite fraction of human plasma proteins designated I+II+III, from which a subfraction designated I+III can be separated [14] may be used as a source of fibronectin and fibrinogen. Essentially, any fraction of human plasma proteins that includes the

fraction I proteins, regardless of the precise method of collection, may be used as a source of fibrinogen and fibronectin.

Thus, side fractions produced and discarded during the manufacture of coagulation factor VIII from cryoprecipitate, which comprise mostly fibrinogen and fibronectin, may also be used. Typical of the processes which may be used to provide useful protein fractions are those described in U.S. Patent No. 4,361,509 (Zimmerman, T. and Fulcher, C. Ultrapurification of factor VIII activity in whole blood or blood plasma. 1983) and in European Patent No. EP 0 416 983 (Burnouf-Radosevich, M. and Burnouf, T. Procede de preparation de concentre du complexe facteur VIII-facteur Von Willebrand de la coagulation sanguine a partir de plasma total.(1991).

It may also be possible to extract fibrinogen and/or fibronectin directly from plasma by, for example, ion-exchange chromatography or polyethylene glycol (PEG) precipitation.

The fibronectin and/or fibrinogen obtained, according to any of the above described methods may optionally be selectively enriched prior to dissolving in the solvent.

It is possible using precipitation to selectively remove fibrinogen from redissolved cryoprecipitate and then precipitate the fibronectin and the remaining fibrinogen from the supernatant. A typical method used is based on that described by Hao, Y.L., Ingham, K.C. and Wickerhauser, M. (1980) Fractional precipitation of proteins with

polyethylene glycol. In "Methods of plasma protein fractionation" (Ed. Curling, J.M.) Pub. Academic Press, London, pp.57-74. Conveniently a first precipitation is carried out with 4% (w/v) polyethylene glycol 6000 and a second with 10% (w/v). Alternatively, any of the standard biochemical procedures for precipitating proteins, such as ethanol precipitation dissolution in alkali (eg. $N_aOH$) or freeze drying the protein precipitate and dissolving it up to a high concentration in solvent, may be used to enhance the fibronectin and/or fibrinogen content of the protein fraction to be used for fibre production.

It is also possible to use heat by taking the redissolved cryoprecipitate of fraction I to +56°C for up to 30 minutes to selectively precipitate fibrinogen by denaturation and then precipitating the fibronectin and remaining fibrinogen comprising fraction from the supernatant with 5% (w/v) polyethylene glycol 6000 at +4°C.

The protein precipitate comprising fibronectin and/or fibrinogen is generally dissolved in the solvent to a concentration of greater than 50mg protein/ml, preferably greater than 70mg protein/ml. However, for some applications such as large scale production of lon fibres more concentrated solutions having a protein concentration greater than 120mg/ml and a high viscosity may be more suitable. If more concentrated protein solutions are required, the precipitate may be freeze-dried first prior to dissolution in the solvent. Preferably the solvent comprises a denaturing agent which acts to denature any

other proteins which may be present in the protein precipitate such as coat proteins of contaminating viruses, while not substantially irreversibly denaturing the fibronectin and/or fibrinogen and/or effecting the fibronectin's and/or fibrinogen's protein properties, such as cell attachment properties. Moreover, this provides a useful virus inactivation step to the process.

A preferred denaturing agent is urea, although other denaturing agents such as guanidine hydrochloride may be employed. Thus, a particularly preferred solvent comprises 2-8M urea, more preferably 2.5-6.5M urea, for example 6M urea. Such a urea solution may be buffered, for example, using Tris in the pH range 7.0-9.0. Since the fibronectin/fibrinogen precipitate contains a significant amount of liquid, dissolution of the precipitate in a solvent containing urea would result in a dilution of the final urea concentration. It is thought that a high concentration of urea may act as a potential virus inactivation step. One method of maintaining a high urea concentration is to add solid urea to a solution in which the protein precipitate is being dissolved to maintain a high concentration. The addition of solid urea must however be controlled to ensure that protein does not precipitate, due to denaturation as a result of high local urea concentrations.

In order to enhance the extrusion properties of the solution and/or alter the properties of the fibre, additives may be added during the dissolution of the

protein. Typically these may include sodium carboxymethyl cellulose, high molecular weight polyethylene glycol and sodium alginate at concentrations for example of 0.1-10% more particularly 1-5% (w/v). Such additives increase the viscosity of the solution and in the case of sodium alginate reduce the self-adhesiveness of the final fibre. Moreover, by including a viscosity enhancer, such as sodium alginate, it is possible to lower the concentration of protein required for fibre production below 50mg/ml. For example it is possible to produce fibres containing 4.6% protein and 4.8% sodium alginate. The apparent viscosity of such a solution at a shear rate of $1007s^{-1}$ was 1310 mPa s (at 23°C).

The solution may be allowed to degas prior to extrusion through the orifice. Degassing the solution serves to prevent air bubbles from becoming entrapped in the fibre during fibre formation.

The orifice through which the solution is extruded may be of any desired size and/or shape. Conveniently the orifice is circular in cross-section with a diameter of between 10μm to 1000μm, preferably 50μm to 500μm, more preferably 100μm to 400μM. However, much larger diameter fibres up to 15mm in diameter may be produced.

Preferably extrusion is carried out at room temperature (e.g. 18-23°C). However, low temperatures (e.g. 4-18°C) may be employed provided that the viscosity of the solution does not increase to a level where extrusion becomes impracticable. If fibrinogen and/or fibronectin

is/are obtained from a cryoprecipitate of plasma and maintained at low temperature (e.g. 4-18°C), the precipitate may be fairly fibrous and difficult to extrude. However, fibrinogen and/or fibronectin obtained from other sources which form gels or non-fibrous precipitates may be extruded at such lower temperatures. Fibres may be extruded over a wide range of extrusion rates. For example, fibres may be extruded at rates between 300µl solution/min to 60ml solution/min. However, the extrusion rate will generally depend upon the number and size of hole the solution is extruded through. Importantly the rate must be such that the protein does not start to precipitate before it is extruded from the orifice.

The coagulation solution into which the protein solution is extruded generally comprises an appropriate combination of acid and salts to cause the protein of interest to coagulate and precipitate out of solution. Coagulation may typically occur over a range of temperatures, preferably between 4°C and 25°C (ie. room temperature).

The isoelectric point for fibrinogen and fibronectin has been recorded in the literature as between pH5 and 6 [15,16]. Surprisingly, however, pH solubility curves show that a mixture of the two proteins in combination, e.g. fibronectin 65%: fibrinogen 35% has a minimum solubility at pH3.0 (see Figure 1). Increasing the proportion of fibrinogen or fibronectin in the mixture results in the minimum solubility for the proteins to move towards pH4.0

(see Figures 2 and 3). The fibronectin only solubility curve is not shown but also shows a movement of the minimum solubility towards pH4.0 - 5.0. However, in all instances a very low pH of <1.0 - 1.5 results in precipitation of the protein(s).

On the basis of this information, substantially pure fibrinogen or fibronectin may be precipitated at a pH <1.0 - 1.5 or between pH 4-6. Fibrinogen/fibronectin mixtures are preferably precipitated at a pH<1.0 - 1.5 or between pH 3-4 (i.e. around the minimum solubility of an appropriate fibronectin/fibrinogen mixture).

Additionally, salts, such as calcium chloride, will precipitate proteins out of solution. Figures 5 and 6 show how increasing concentrations of calcium chloride affects the solubility of fibrinogen/fibronectin solutions. Calcium chloride increases the ionic strength of the solution and leads to an increase in protein precipitation. Addition of calcium chloride also decreases the pH due to the production of hydrochloric acid.

Thus, fibrinogen and/or fibronectin may be precipitated from solution by appropriate pH control, high salt or preferably a combination of the two. The importance of combining both salts and pH is shown in Figure 9 where the highest level of protein precipitation is seen where there is both a decrease in pH and an increase in ionic strength.

Preferred coagulation solutions for the formation of fibronectin and/or fibrinogen fibres are strong acids at low pH (<pH1.0-1.5) such as hydrochloric acid or sulphuric acid at concentrations greater than 0.05M, generally greater than 0.1M, or 0.2M, optionally with the addition of salts such as calcium chloride or sodium sulphate between 1-10%, preferably 2-7%. Particularly preferred solutions are 0.25M HCl /2% (w/v) $CaCl_2$ and 0.05M $H_2SO_4$/1% (w/v) $Na_2SO_4$.

Alternatively, weaker acids such as citric acid and acetic acid between 5-20% with higher levels of salts 5-20% such as sodium citrate and sodium chloride are more suitable for precipitation around the minimum solubility of fibronectin/fibrinogen mixtures (i.e. pH 1.5 - 4.0). Particularly preferred examples are 5-10% acetic acid with 10% sodium chloride, and 1M citric acid/1M tri-sodium citrate, with 2-10% calcium chloride, pH varying from 2.0-0.8 depending on calcium chloride concentration.

Alternative material which may be used in coagulation solutions include non-ionic polymers, such as dextran and polyethylene glycol (eg. 25-50% PEG, pH 4.3-4.8), organic solvents, such as ethanol or acetone, ionic polyelectrolytes, such as carboxymethylcellulose, and appropriate combinations of ions and salts such as tri-sodium citrate, sodium sulphate or ammonium sulphate.

Once formed the fibre or fibres may be drawn out of the coagulation solution, preferably through a washing solution to remove contaminants such as acid or salts.

Isotonic solutions, buffered saline solutions such as phosphate buffered saline, or water may be employed as the washing solution. The strength and pH of the washing solution is not critical for fibre formation, but a suitable solution has been found to be 0.01M phosphate-buffered saline, pH 7.4 for fibres with diameters up to 200μm. For larger diameter fibres 200mM tris-HCL, pH7.6 was found to be appropriate. Neutralisation of a fibre took about 15 minutes for a fibre 1cm long and diameter 5mm, whilst fibres with dimensions 10cm long and diameter 1.5cm were successfully washed after 30mins. Once washed, the fibres may be further dehydrated, if required, by immersion in a solvent or solvent/water bath. Suitable solvents include acetone or ethanol.

It is possible to carry out an essentially continuous fibre producing process once fibre formation has commenced, by appropriate "topping-up" or replacement of the protein solution, and/or coagulation solution. In this manner, tens of centimetres, metres or even hundreds of metres of protein fibre may be produced per run. For example, 70cm of a 1mm wet-fibre may be produced from 1ml of a solution comprising 100mg fibrinogen/fibronectin and 1% alginate when spun from a 350μm diameter needle. Fibronectin/fibrinogen alone or optionally including 0.5% carboxymethylcellulose yields 120-180cm of a 200-600μm fibre. Additionally such fibres may be further drawn to reduce their diameter.

Continuous fibres may be formed when a solution containing 140mg/mL total protein and 1.3% sodium alginate are spun through a capillary, length 18mm and diameter 1mm, into a coagulation bath containing 0.25 M hydrochloric acid and 2% $CaCl_2$, pH < 1.0. Alginate containing solutions are shear thinning, that is they display a decrease in apparent viscosity as they are sheared. The shear on the fluid in the capillary is proportional to $1/r^3$ where r is the radius of a spinneret or capillary orifice. Thus as the radius of the orifice decreases, the shear rate in the capillary increases by a large amount and there is a consequent decrease in the apparent viscosity of shear thinning solutions. High viscosities are generally required for wet spinning solutions, so a capillary with a wide diameter, eg 1mm, may be used to spin fibres. The calculated shear rate in an 18mm long, 1mm diameter capillary is $1358s^{-1}$ and the 140 mg/mL protein/1.3 % sodium alginate solutions had an apparent viscosity of 413 mPa s at a shear rate of $1007s^{-1}$ and at 23°C. Thus solutions with an apparent viscosity of this order may be extruded through the capillary described to form continuous fibres. A long capillary may be used to increase the residence time of the solution in the spinneret and thus orientate the protein molecules in the solution, in preparation for coagulation into orientated protein fibres.

By increasing the protein concentration/viscosity of the solutions further, spinnerets with a smaller diameter orifice (with or without a capillary) may also be used.

The fibre is generally stabilised by drying in air or subjecting the fibre to an appropriate freeze-drying regime [17]. Severe drying of the fibre such as by freeze-drying is preferred since it is believed that this may increase cross-linking in the fibre and/or alter porosity. The fibre may be further processed. For example metal ions such as copper and/or zinc may be incorporated, typically in micromolar concentrations. This may be achieved by soaking the fibre in a solution of a metal salt. Inclusion of such salts has been observed to improve the stability of fibronectin in materials comprising fibronectin in *in vitro* experiments and alter the cell adhesion/migration properties of the material. Different cell types appear to be tolerant to different concentrations of metal ions in the material. Thus, incorporating different concentrations of metal ions into different regions of a fibre may lead to cell segregation by cell-type exclusion.

The fibre may be cut into appropriate lengths preferably when wet using a sharp edge and optionally supported on a substrate such as alginate gel or sheet, hyaluronate films, collagen gels/sponges, agar/agarose sheets or gelatin sponges as well as a variety of synthetic matrices such as polymer matrices in order to provide a composite material. The fibres may be generally contacted with the substrate while they are wet and naturally adhesive. The fibres may be lain across a flat substrate, eg. collagen sponge, or wound onto a roller covered with

the substrate. Gamma irradiation and/or chemical cross-linking agents may be used to increase the strength of fibre adhesion to the substrate, if required. Gamma irradiation may also provide an important viral inactivation step. As an example, samples have been exposed to 48hrs irradiation with a maximum total exposure of approximately 3 MR in order to sterilise material for *in vitro* cell culture.

The composite material comprising fibre(s) supported on a substrate may be used to promote wound healing and/or ordered regeneration of damaged tissue. The substrate should be a physiologically compatible material and may be a biodegradable or resorbable material, such that it may be left in the wound without substantial deleterious effect to the host organism. Preferably the substrate is also non-toxic and/or non-antigenic. A typical substrate is a collagen sponge, thickness 1-5mm, with a pore size range of 30-1000$\mu$M, porosity or void volume 30-85% which may be non-cross-linked or chemically cross-linked for stability may be used as a substrate.

In another aspect, the present invention provides a fibre comprising fibrinogen/fibronectin for use in therapy. The present invention further provides a fibre comprising fibrinogen/fibronectin in the manufacture of medicaments, dressings or devices for use in therapy. The fibres typically comprise fibrinogen/fibronectin in the ratio 9:1 to 1:9, preferably 7.5:2.5 to 2.5:7.5, more preferably 6.5:3.5 to 3.5:6.5. A particularly preferred fibre

comprises fibrinogen/fibronectin in the ratio 3.5:6.5. The fibre comprises many substantially parallel aligned fibrils of fibrinogen/fibronectin and each fibre is generally 10μm to 1000μm in diameter, preferably 50μm to 500μm, more preferably 100μm to 400μm comprising parallel microa-diameter fibrils. Without wishing to be bound by theory, it is thought that the fibronectin and fibrinogen are complexed so as to form a composite fibre. The fibre may be tens of centimetres long, even metres or hundreds of metres long and can be provided such that the fibre may be cut to an appropriate size prior to use.

The fibres may be used to prepare a mat comprising any number of fibres which may be generally aligned in parallel or interwoven in a criss-cross manner. Additionally, the fibres may be used to form a 3-dimensional matrix structure.

The fibres may be twisted in order to improve tensile strength, this may be important where the fibres are not attached to a substrate. Without wishing to be bound by theory, it is thought that twisting the fibre, which leads to a significant increase in maximum tensile strength, results in the presentation of previously remote binding sites of fibronectin to each other and the enhancement of cross-linking within the fibre. Electromicrograph studies of such fibres show that untwisted fibres have a non-compacted centre in contrast to twisted fibres which display a compacted centre. Typically, untwisted fibres may possess a tensile strength in the range 5-39 $N/mm^2$

depending on the fibres composition and method of formation while twisted fibres may display a tensile strength in the range 40-85 $N/mm^2$. Preferably the twisting of fibres is carried out immediately after fibre formation but before the material loses contacting properties, such as adhesive properties.

The fibres and/or fibre coated substrate may be used as, or as part of, a wound dressing, or are applied to open wounds separately from a conventional dressing, used for guided nerve regeneration, guided tendon/ligament repair and the like. To derive improved strength and/or cosmetic acceptability of the mature wound it is preferred that the fibre and/or fibre coated substrate are applied to the wound with the fibres aligned with features of the surrounding tissue so as to encourage invasion in an ordered manner along a longitudinal direction of the fibres.

It will be appreciated that the ratio of fibrinogen and fibronectin in a fibre may be controlled within the limits defined herein, for particular applications. For example it has been demonstrated that fibres comprising fibrinogen/fibronectin in the ratio 1:1 show an increased *in vitro* cell migration rate for four cell types tested (rat schwann cells, rat tendon, rat skin fibroblasts and human dermal fibroblasts) compared to higher proportions of fibronectin or fibrinogen. Fibronectin is known to be important for cell adhesion. Without wishing to be bound by theory it is thought that the effective "dilution" of the

fibronectin's cell adhesion properties with fibrinogen allows cells to adhere to the fibre, but not so strongly that they are unable to migrate along the protein fibre. A 1:1 ratio of fibrinogen/fibronectin therefore appears to provide a good balance between migration and adhesion.

Additionally, fibronectin/fibrinogen films can be made by mixing the protein solution and coagulation solution, casting or moulding and allowing the mixture to dry.

The present invention will now be further described by way of example with reference to the following non-limiting examples section and accompanying figures, wherein:

Figure 1 shows the solubility curve for a solution comprising fibronectin/fibrinogen in a 6.5:3.5 ratio.

Figure 2 shows the solubility curve for a solution comprising fibronectin/fibrinogen in a 2.5:7.5 ratio.

Figure 3 shows the solubility curve for a substantially pure fibrinogen solution.

Figure 4 shows the solubility curve for a solution comprising fibrinogen/fibronectin in a 3.5:6.5 ratio as the pH of the solution decreases from 7.8 to 4.1 with increasing concentrations of calcium chloride;

Figure 5 shows the solubility curve for a solution comprising fibrinogen/fibronectin in a 7.5:2.5 ratio as the pH of the solution decreases from 7.3 to 4.6 with increasing concentrations of calcium chloride.

Figure 6 shows solubility curves for a 5mg/ml protein solution of 67% fibronectin and 33% fibrinogen as influenced by the addition of various salts;

Figure 7 shows the solubility of fibronectin/fibrinogen (5mg/ml:67%/33%) with increasing concentrations of ammonium sulphate;

Figure 8 shows the solubility of fibronectin/fibrinogen (5mg/ml:67%/33%) with the addition of ethanol or polyethylene glycol 4000; and

Figure 9 shows how the solubility of fibronectin/fibrinogen (5mg/ml:67%/33%) is altered when both pH and ionic strength of the solution are altered.

Figure 10 shows schematically a pilot scale rig which was used to prepare fibres of the present invention.

Example 1: Preparation of fibrinogen/fibronectin precipitate.

A fibrinogen/fibronection precipitate was prepared as described previously in Foster, P.R., Dickson, A.J., McQuillan, T.A., Dickson, I.H., Keddie, S. and Watt, J.G. (1982); Control of large-scale plasma thawing for recovery of cryoprecipitate factor VIII. Vox Sanguinis, 42: 180-189.

Example 2: Selective Enrichment of fibronectin by PEG precipitation.

Frozen cryoprecipitate was thawed overnight at 4°C, shredded into small pieces and dissolved in 5 times (w/v) phosphate-buffer (0.01M $Na_2HPO_4$, 0.01M $NaH_2PO_4.2H_20$, 0.015M NaCl, pH 7.0) at 37°C. When dissolved the solution contained 16-18 mg/mL total protein of which 75-80% was fibrinogen and 20-25% was fibronection.

The solution was allowed to cool to room temperature and 50% (w/v) PEG 4000 was added to give a final concentration of 4% (v/v). The solution was stirred briefly and left for 1 hour at room temperature to precipitate the fibrinogen present. The precipitate was separated from the solution, which contained the fibronectin, by centrifugation for 15 minutes at 4500 r.p.m. The fibrinogen precipitate was discarded and the volume of the remaining supernatant measured. The supernatant contained fibronection and fibrinogen in a 2:1 ratio.

The concentration of PEG 4000 in solution was increased to 10% (v/v), stirred briefly, and left either overnight or for 1 hour at room temperature to precipitate out the fibronectin. The solution was then centrifuged for 15 minutes at 4500 r.p.m. to produce a fibronectin-rich pellet. The pellet contained both fibronectin and fibrinogen in the ratio 2:1. The pellets were stored frozen at -20°C until required.

Further enrichment of the solution could be carried out be redissolving the fibronectin pellet and repeating both the 4% and 10% PEG cuts to remove more fibrinogen and reprecipitate the fibronectin.

Example 3: Extrusion of fibrinogen/fibronectin fibres.

The protein precipitate comprising fibronectin and fibrinogen in a 2:1 ratio as well as polyethylene glycol from the precipitation step described above and other plasma proteins in small proportions such as albumin (not determined) was dissolved in 6M urea to give a final total protein concentration of >70mg/ml.

Sodium alginate was added to a concentration of 1% in order to increase viscosity and reduce the self adhesiveness of the final fibre.

The resulting viscous solution was then allowed to degas and passed through a 350$\mu$m diameter orifice into a coagulation bath whereupon a white fibre was formed. The fibre was then drawn out of the coagulation bath (pH<1.0, 0.25M HCL, 2%(w/v) calcium chloride at room temperature) and washed in 0.01M phosphate-buffered saline pH7.4, before being left to dry at room temperature.

Example 4: Determination of optimal coagulation conditions.

The PEG precipitate as described according to Example 3 was dissolved in phosphate buffer up to a concentration of 5mg/ml without the addition of sodium alginate and the protein solution subjected to solubility experiments.

Figures 1 to 5 show the % protein remaining in solution after altering either the pH or the ionic strength of the solution with calcium chloride. Protein in solution was measured by the Bradford dye binding assay (Bio-Rad, Munich, Germany). Dashed lines on Figures 1-3 represent

the decrease in protein solubility as the pH is lowered to less than 1.5 or 1.0.

Figures 1 and 2 show the minimum solubility of the fibronectin/fibrinogen mixture to be around pH3.0 and pH4.0 respectively and Figure 3 shows the minimum solubility of fibrinogen to be around pH4.0.

Figures 4 and 5 show that increasing concentrations of calcium chloride reduce the solubility of fibronectin/fibrinogen in solution.

Figures 6,7 and 8 show that increasing concentrations of salt, ammonium sulphate, ethanol and PEG reduce the solubility of fibronectin/fibrinogen in solution.

Figure 9 shows the effect of altering both pH and ionic strength on fibronectin/fibrinogen solutions.

After determining the influence of pH and salt concentration on fibronectin/fibrinogen solubility various compositions of coagulation baths were tested in relation to fibre formation. A summary of these results is presented in Table 1.

| Acid/Salt combination | pH range | Comments |
|---|---|---|
| Acetic acid/Sodium chloride | 1.7-2.5 | Precipitation but no stable fibres when acetic acid concentrations between 1 and 10% (v/v) used and with salt concentrations 0-5% (w/v). Improved fibre formation with 5-10% acetic acid and 10% salt concentration (pH 2.0-1.7) |
| Sulphuric acid/sodium sulphate | 1.0-1.8 | Stable fibre formation with 0.05M sulphuric acid and at least 1 (w/v) sodium sulphate. Increasing the acid strength reduced the amount of salt required for precipitation. |
| Citric acid/Tri-sodium citrate | 3.0 | 0.1M-1.0M tri-sodium citrate/citric acid with and without 2% calcium chloride gave good precipitation but fibres were difficult to handle. |
| Hydrochloric acid/Calcium chloride | 0.4-1.5 | With no salt an acid concentration >0.1M is required for good precipitation with fibre formation being improved on the addition of at least 2% calcium chloride. For higher acid concentrations less salt is required for fibre formation. 0.25M HCI/2% Calcium chloride were chosen as the preferred conditions so as to minimise the concentration of both acid and salt whilst achieving good fibre formation. The protein is denatured during this procedure. |

Table 1. Summary of wet spinning conditions. Dope is 65% fibronectic : 35% fibrinogen, with protein concentration ≥ 70mg/mL. All trials carried out at room temperature

It can be seen from these results that fibronectin/fibrinogen will generally precipitate out of solution at low pH,<pH1.5-1.0, using HCL/$CaCL_2$ and $H_2SO_4/Na_2SO_4$ combinations and around the pH 3.0-4.5 using weaker acids, citric acid/citrate and asetic acid/NaCL combinations.

Example 5: Tensile Strength Testing of Fibronectin/Fibrinogen Fibres.

A solution of fibronectin/fibrinogen was prepared according to Example 1, with total protein, 5mg/ml made up of 3.4mg/ml fibronectin/1.3mg/ml fibrinogen and 0.5mg/ml albumin. The protein was precipitated at room temperature by adding 0.1M citric acid, pH3.0-3.5 to the protein solution in a 2:1 (vol/vol) ratio of protein to acid. The resulting pH of the solution was 4-4.5. The solution was gently stirred to allow the protein precipitate to aggregate and protein strands were drawn slowly from the precipitate using a fine tipped glass rod.

Drawn and in some instances twisted, protein fibres of length approximately 2cm and diameter 100-800$\mu$m were attached to a metal frame with polystyrene cross bars, using cyanoacrylate adhesive, (RS Components Ltd., Corby, UK). To prevent sample contamination with the adhesive, the strands were glued to the frame in a horizontal position and the glue allowed to dry before testing so that no glue could run down the specimen. The length of sample between the two points of attachment was then measured and

taken as the sample gauge length. Samples were then tested in a dry state using a Testometric 220M tensile testing rig (The Tensile Testing Co. Ltd., Roshdale, UK) operating at a strain rate 2mm/min. To test the in-strand variation in strength, tested samples were splinted and then retested ensuring that the point of breakage was clear of the glued region. Specimen dimensions were determined using a Nikon Profile Projector. Bulk protein material was made by compressing and dewatering protein precipitate before cutting it into 2mm width strips. Moist samples were tested in an enclosed damp environment by surrounding the testing frame with a plastic cylinder containing damp paper. The relative humidity was measured with a portable probe hygrometer, (RS Components) and the moisture content of the material was also calculated using a dry weight method.

During tensile testing of wet specimens (>40% moisture content) it was found that the application of a constant strain rate, 2mm/min and a constant relative humidity (>60%), resulted in extrusion of the material. The strand length was found to increase by up to 7-fold with a consequent decrease in diameter. Thus the strands may be drawn down to their required diameter once they are formed, provided that the relative humidity is at least 60%.

Results for tensile strength testing of dried specimens are given Table 2. The tenacity for the twisted strands was calculated as 2.5-3.5g/denier in their dry state.

Twisting the fibronectin material leads to a significant increase in strand maximum tensile strength probably due to the presentation of previously remote binding sites to each other and thus the enhancement of cross linking within the strand. Electromicrograph studies have shown that the plain strand has a non-compacted centre whilst the twisted strand shows a compacted centre which is associated with the increased tensile strength from 15 to 61 $N/mm^2$. It should be noted that twisting must occur immediately after strand formation before the material loses its adhesive properties. Defects in the strands were observed when twisting had been carried out after the loss of self-adhesion ability resulting in holes in the material and a lower tensile strength. Twisted material without defects appeared glassy whilst those with defects were duller and bubbles could be viewed even under the x10 magnification of a light microscope.

The site of breakage for the twisted strands was typical of that of a brittle material, i.e. a clean surface. The twisted fibronectin/fibrinogen strands have an average tensile strength of $61N/mm_2$ lower than that of collagen fibres. This is to be expected since collagen is found in tissues which are load bearing, e.g. tendons and ligaments. The orientation of the strands caused by the micron-diameter fibrils gives them a greater tensile strength compared to the bulk material, $22.5N/mm^2$. The bulk material has no overall orientation and thus could have reduced intermolecular bonding.

TABLE 2

The maximum tensile strength and elongation at break of dried fibronectin/fibrinogen conjugate strands.

| Material | Maximum tensile strength ($N/mm^2$) ± st.dev | elongation at break (%) |
|---|---|---|
| Bulk | 22.5±4.9 | 2.7 |
| Non-twisted strands | 15.0±2.6 | 4.9 |
| Twisted Strands | 60.9±14.4 | 3 |
| Twisted strands with identified defects | 21.5±8.6 | |

Example 6: Wet spun fibres using test rig and fibre properties

A suitable spinning dope was prepared by dissolving 281 g of freshly thawed protein/polyethylene glycol precipitate in 100 mL of 6 M urea. Sodium alginate (4.0 g) was also added to give a solution with a final volume of approximately 310 mL and a final urea concentration of 2 M. The final protein concentration was 140 mg/mL total protein, consisting of 50% fibronectin and 50% fibrinogen combined with 1.3% (w/v) sodium alginate. During dissolution temperature was maintained at 37°C using a water bath and agitation was provided by a single impeller mixer stirring at 500 - 700 rev/min. Dissolution occurred over 4 - 5 hours and the dope was left overnight at room

temperature to degas.

Fibres were then prepared using the pilot rig as schematically shown in Figure 10.

The prepared solution was placed in a stainless steel reservoir (1) and transferred under nitrogen pressure (15 - 40 p.s.i) through a polythene tube (3), diameter 1 cm, to a flow control valve (5). When the polythene tube (3) had filled, the valve (5) was opened and the dope passed through to a 1 mL/rev. geared metering pump (7) (Slack & Parr, Kegworth, UK). Dope was pumped at a rate of 10 - 20 mL/min to the spinneret (9). The spinneret (9) had an orifice diameter of 1 mm and a channel length of 18 mm. The dope was extruded through this into a coagulation bath (dimensions 10 x 180 cm) containing 0.25 M Hcl, 2% $CaCl_2$, pH<1.0.

The formed fibre (13) was initially drawn by hand along the length of the coagulation bath and attached to steel drawing rollers (15) (diameter 10 cm, speed of rotation 21 rev/min) which picked the fibres from the coagulation bath and transferred them to a washing bath (17), for acid neutralisation. A second set of collecting rollers (19) (diameter 10 cm, speed of rotation 31 rev/min) were used to take some of the fibres from the washing bath (17) to a dehydrating bath (21) containing 100% acetone and also to draw the fibres down to a finer diameter.

Measurements of tensile strength of the fibres wet spun on the pilot scale rig were made and results are shown in Table 3.

The average diameter for the 14% protein/1.3% alginate fibres was 540 ± 63 and 563 ± 33μm with and without acetone treatment respectively. Comparative results for the 4.6% protein/4.8% alginate fibres were 415 ± 36 and 635 ± 17μm. Results are given ± 95% confidence intervals.

When the surface of fibronectin/fibrinogen cables drawn from a 5mg/mL solution was examined using scanning electron microscopy, it was found to consist of parallel micro-diameter fibrils, which are believed to provide the contact guidance cues for the alignment of cells on top of the drawn cables. Examination of the surface of spun fibres revealed ridges approximately 10μm wide aligned along the longitudinal axis of the 4.6% protein / 4.8% alginate fibres. The 14% protein / 1.3% alginate fibres revealed approximately micron-wide ridges when examined under a high magnification.

| Protein conc(%) | Alginate conc(%) | Temp (°C) RH(%) | Tensile strength ($N/mm^2$) | Set(%) | Elongation at break (%) | Acetone treated |
|---|---|---|---|---|---|---|
| 14.0 | 1.3 | 24-25 59-67 | 28.1±9.2 (11) | 20.5±6.5 (11) | 52.4±14.3 (11) | yes |
| 14.0 | 1.3 | 24-25 60.70 | 7.5±2.0 (9) | 11.8±7.2 (8) | 43.1±11.8 (9) | no |

Table 3. Tensile properties of wet spun fibronectin/fibrinogen fibres. Tensile strength was measured as the maximum load required to fracture the specimen per unit cross sectional area. Set is the permanent increase in sample length following breakage, expressed as a percentage of the original length. Elongation at break is the % increase in sample length at the time of fracture, expressed as a % of the original length. Number of samples tested for each group is given in brackets and all results are ±95 confidence intervals.

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## CLAIMS

1. A process for the preparation of a protein fibre comprising fibronectin and/or fibrinogen, comprising the steps of:
   - adding fibronectin and/or fibrinogen to a solvent so as to form a fibronectin and/or fibrinogen solution; and
   - extruding the solution through an orifice into a coagulation solution so as to form said protein fibre.

2. A process according to claim 1 wherein the protein fibre comprises fibronectin and fibrinogen.

3. A process according to claim 2 wherein the fibronectin and fibrinogen are added as a solid and wherein the ratio of fibronectin to fibrinogen is in the range 9:1 to 1:9.

4. A process according to claim 3 wherein the ratio of fibronectin to fibrinogen is in the range 7.5:2.5 to 2.5:7.5.

5. A process according to claim 3 wherein the ratio of fibronectin to fibrinogen is 6.5:3.5.

6. A process according to claim 3 wherein the ratio of fibronection to fibrinogen is 1:1.

7. A process according to any preceding claim wherein the fibronectin and/or fibrinogen is obtained from a cryoprecipitate of blood plasma.

8. A process according to any preceding claim wherein the fibronectin and/or fibrinogen is dissolved in the solvent to a concentration of greater than 50 mg protein/ml.

9. A process according to claim 8 wherein the fibronectin and/or fibrinogen is dissolved in the solvent to a concentration of greater than 120mg protein/mL.

10. A process according to any preceding claim wherein the solvent comprises a denaturing agent which acts to denature any other proteins which may be present while not substantially irreversibly denaturing the fibronectin and/or fibrinogen.

11. A process according to claim 10 wherein the denaturing agent is 2-8M urea.

12. A process according to any preceding claim further comprising addition of an additive to the fibronectin and/or fibrinogen solution, the additive increasing the viscosity of the solution and/or reducing self-adhesiveness of the fibre.

13. A process according to claim 12 wherein the additive is selected from sodium carboxymethyl cellulose, high molecular weight polyethylene glycol and/or sodium alginate.

14. A process according to any preceding claim wherein the orifice through which the solution is extruded is circular in cross-section with a diameter of 10 μm to 15mm.

15. A process according to any preceding claim wherein the fibre is extruded at rates between 300 μl solution/min to 60 ml solution/min.

16. A process according to claim 1 for the preparation of a substantially pure fibrinogen or fibronectin fibre wherein the coagulation solution is at a pH<1.0 - 1.5 or between pH4 - 6.

17. A process according to any preceding claim for the preparation of a fibronectin/fibrinogen fibre wherein the coagulation solution is at a pH<1.0 - 1.5 or between pH3 - 4.

18. A process according to any preceding claim wherein the coagulation solution comprises acid and/or salt.

19. A process according to claim 18 wherein the acid is selected from hydrochloric acid, sulphuric acid, citric acid and acetic acid.

20. A process according to claim 18 wherein the salt is selected from calcium chloride, tri-sodium citrate, sodium sulphate and/or ammonium sulphate.

21. A process according to claim 18 wherein the coagulation solution is selected from
0.25M HCl/2% (w/v) $CaCl_2$;
0.05M $H_2SO_4$/1% (w/v) $NA_2SO_4$;
5-10% acetic acid/10% NaCl; and
1m citric acid/1M tri-sodium citrate/2-10% $CaCl_2$

22. A process according to any proceeding claim for producing a fibre greater than 10 cm in length.

23. A fibre comprising fibrinogen/fibronectin for use in therapy.

24. A fibre according to claim 23 comprising fibrinogen/fibronectin in the ratio 9:1 to 1:9.

25. A fibre according to claim 24 comprising fibrinogen/fibronectin in the ratio 7.5:2.5 to 2.5:7.5.

26. A fibre according to any one of claims 23-25 comprising many substantially parallel aligned fibrils of fibrinogen/fibronectin.

27. A fibre according to claim 26 which is 10 μm to 15mm in diameter.

28. A fibre according to any one of claims 23-26 greater than 10 cm in length.

29. A fibre according to any one of claims 23-28 which is twisted.

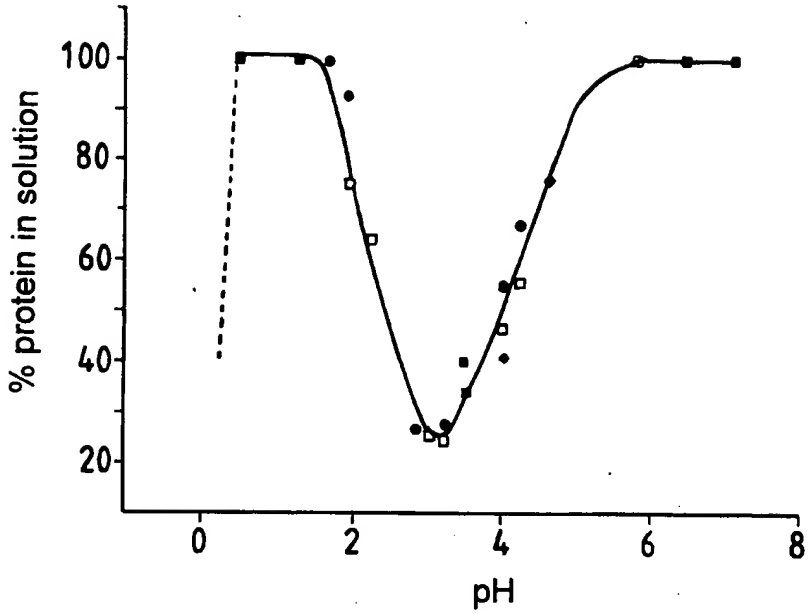
30. A fibre according to any one of claims 23-29 for use in therapy.

31. A composite material comprising a fibre or a fibre prepared according to any preceding claim together with a support substrate.

32. A composite material according to claim 31 for use in wound healing and/or regeneration of damaged tissue.

33. A mat comprising any number of fibres according to any one of claims 22-29, wherein said fibres are generally aligned in parallel or interwoven in a criss-cross manner.

Fig. 1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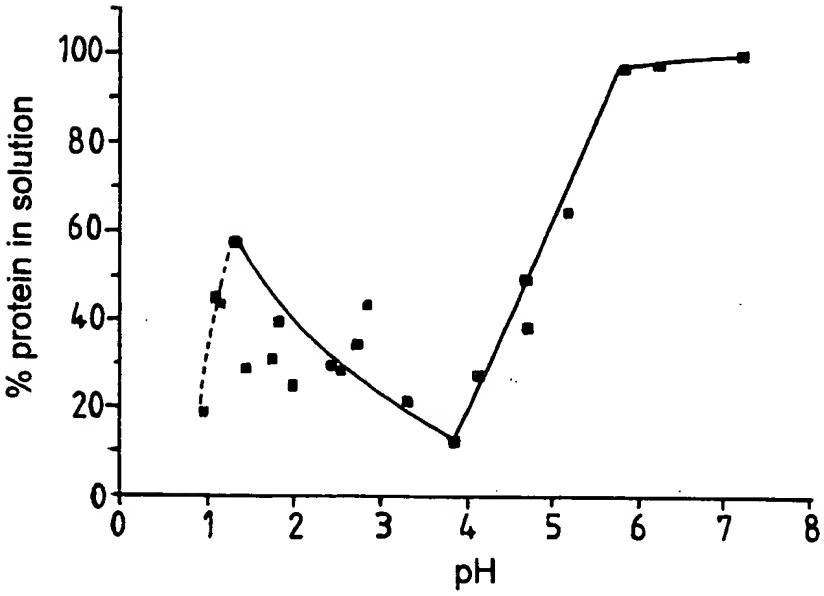
6
8
pH

Fig. 2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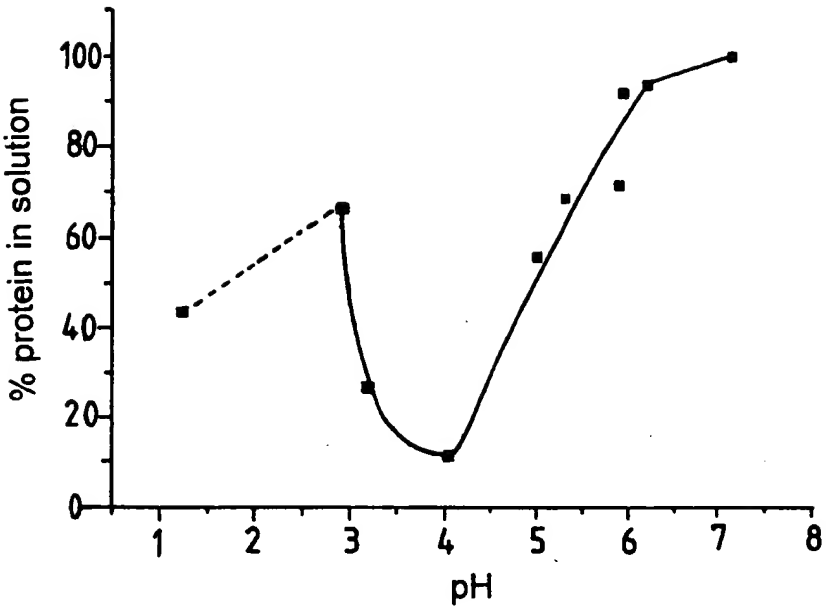
pH

Fig. 3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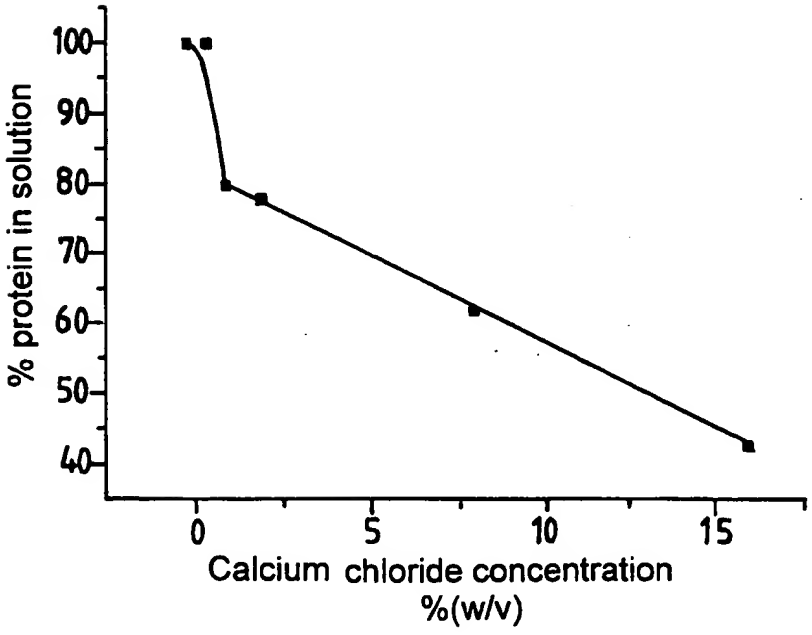
pH

Fig. 4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Calcium chloride concentration
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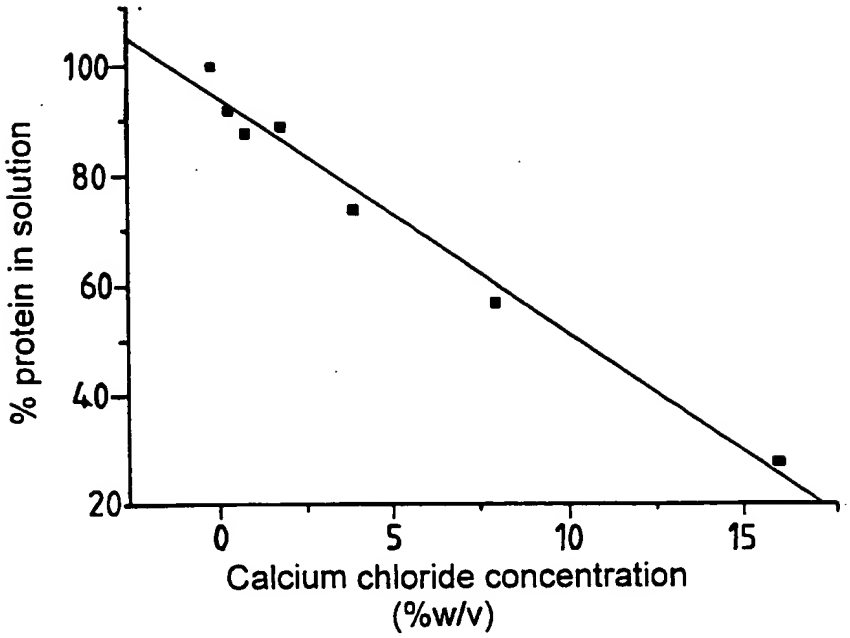
Fig. 5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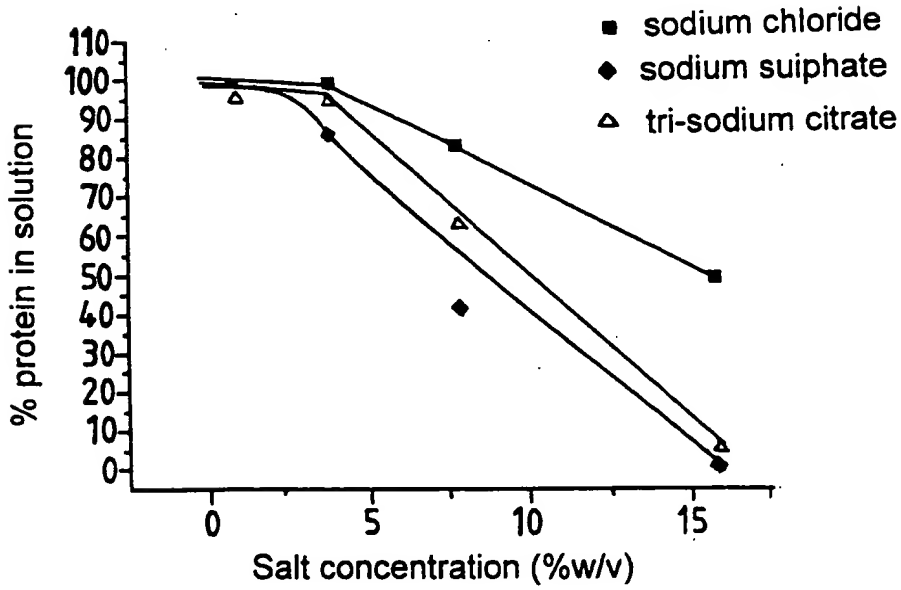
Calcium chloride concentration
(%w/v)

Fig. 6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sodium suiphate
tri-sodium citrate
% protein in solution
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50
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10
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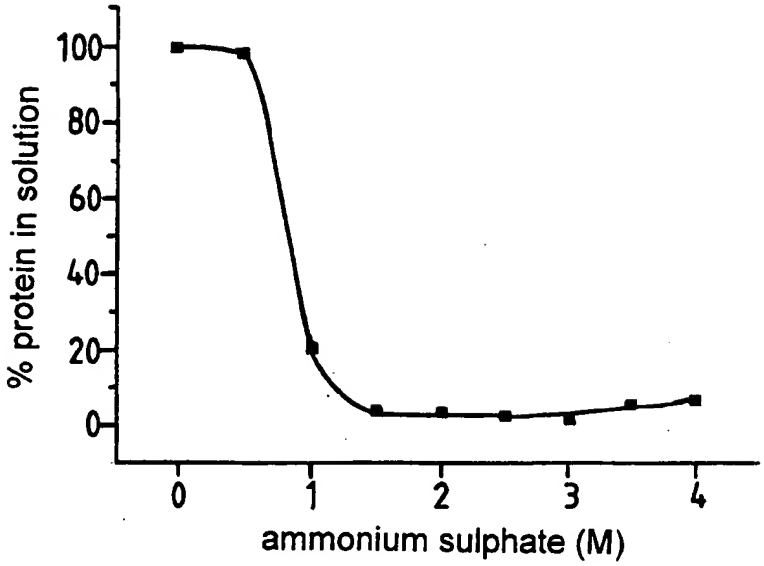
0
5
10
15
Salt concentration (%w/v)

Fig. 7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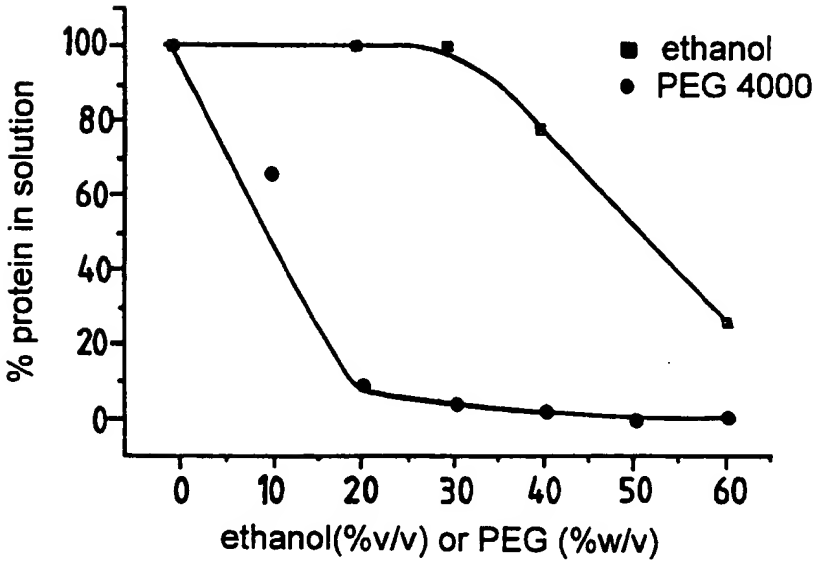
1
2
3
4
ammonium sulphate (M)

Fig. 8


% protein in solution
100
80
60
40
20
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10
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60
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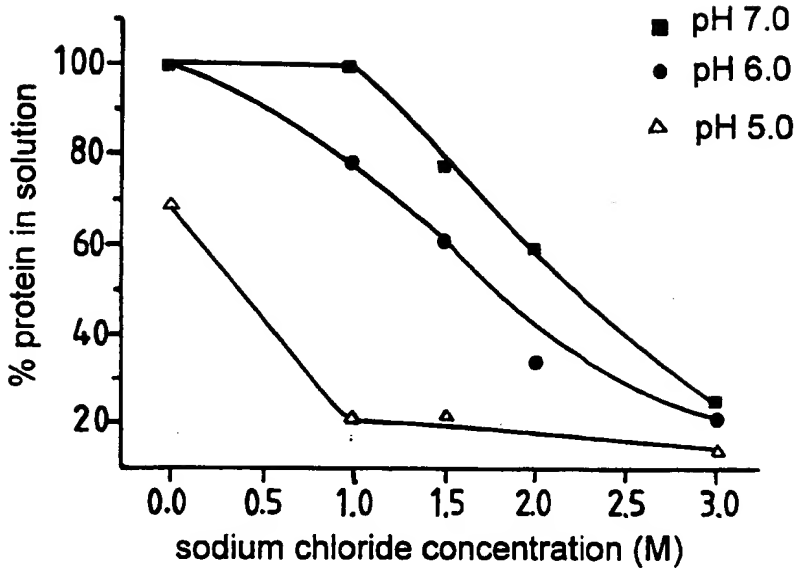
PEG 4000

Fig. 9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pH 5.0
% protein in solution
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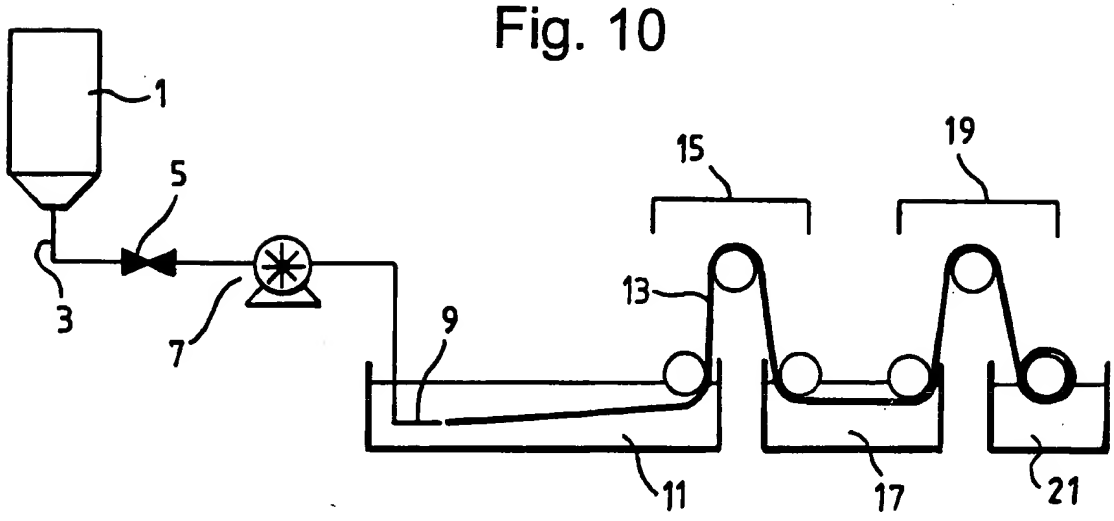
2.5
3.0
sodium chloride concentration (M)

Fig. 10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# INTERNATIONAL SEARCH REPORT

Ir ational Application No
PCT/GB 98/03531

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 D01F4/00 A61L15/32

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 D01F A61L

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

| Category ° | Citation of document, with indication, where appropriate, of the relevant passages | Relevant to claim No. |
|---|---|---|
| A | WO 92 13003 A (UNIV LONDON) 6 August 1992<br>see the whole document | 1-33 |
| A | US 4 973 466 A (REICH CARY)<br>27 November 1990<br>see the whole document | 1-33 |

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|---|---|
| 9 March 1999 | 18/03/1999 |

| Name and mailing address of the ISA | Authorized officer |
|---|---|
| European Patent Office, P.B. 5818 Patentlaan 2<br>NL - 2280 HV Rijswijk<br>Tel. (+31-70) 340-2040, Tx. 31 651 epo nl,<br>Fax: (+31-70) 340-3016 | Tarrida Torrell, J |

1

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# INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No
PCT/GB 98/03531

| Patent document cited in search report | Publication date | Patent family member(s) | Publication date |
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| | | AU 1172292 A | 27-08-1992 |
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| | | EP 0567508 A | 03-11-1993 |
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| US 4973466 A | 27-11-1990 | US 5124155 A | 23-06-1992 |